CERTIFICATE OF MAILING

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12 Nov. 1982
Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Charles D. Jones	
Serial No.	:	331,042	Group Art Unit: 121
Filed	:	December 16, 1981	
For	:	ANTIESTROGENIC AND ANTI-ANDROGENIC BENZOTHIOPHENE	Examiner: R. Schwartz
Docket No.	:	X-5526A	

DECLARATION UNDER 37 C.F.R. 1.132

Blake L. Neubauer, Ph.D., declares as follows:

I earned my doctorate in pharmacology and toxicology at West Virginia University in 1979, and then went to the University of Colorado for two years of post-doctoral fellowships in the anatomy department and in the pharmacology department, School of Pharmacy. I became employed at Eli Lilly and Company in 1981, and now am a research scientist in the central nervous system and endocrinology research division of Lilly Research Laboratories. I am responsible for carrying out research on the effects of compounds having estrogenic and anti-estrogenic activity on accessory sex organs. I am an author of about 13 scientific articles, and of about 5 articles which are now in press.

Male Guinea Pig Responses

I have directly supervised the testing of two compounds related to the above-named patent application, and this paper reports the results of these tests. The two compounds which are here compared are 6-hydroxy-2-(4-hydroxyphenyl)-3-[4-(2-piperidinoethoxy)benzoyl]benzo[b]thiophene (compound I) and 6-hydroxy-2-(4-hydroxyphenyl)-3-[4-(2-pyrrolidinoethoxy)benzoyl]-benzo[b]thiophene (compound II). Compound I was tested in the form of its hydrochloride salt, and compound II was the free

base. I recognize that it is conceivable that the comparison between the two compounds is thus not precise, but the data are reported here because they are the only available results of testing these compounds in male guinea pigs.

The tests were carried out as follows. Sexually mature male Hartley guinea pigs weighing 450-550 grams were obtained from a standardized source, and were castrated via the scrotal route under metophane anesthesia. Animals were injected subcutaneously with compound I or compound II in 0.2 ml. of corn oil daily for 14 days. Control animals received an equal volume of corn oil. The animals were then sacrificed by decapitation, and the seminal vesicles were removed. The seminal vesicles were then separated into the epithelial (SVE) and fibromuscular stromal (SVM) tissue components and weighed.

Each treatment group consisted of six guinea pigs. Each of the test compounds was administered at six different dosages, as shown in the table below. The body weights, tissue weights, and normalized tissue weights, expressed as milligrams per 100 grams of body weight, are reported in the table below as the mean and standard error of each treatment group.

TABLE I

COMPOUND II

Treatment	Body Weight (gm)	SVM Weight (mg)	Normalized SVM Weight	SVE Weight (mg)	Normalized SVE Weight
Control	512.7 ± 21.5	169.7 ± 15.9	28.8 ± 3.2	36.3 ± 2.7	7.9 ± 0.7
Compound II 0.0002 mg/kg	491.0 ± 7.5	187.0 ± 23.2	32.0 ± 4.1	36.7 ± 2.3	7.1 ± 0.3
Compound II 0.002 mg/kg	518 ± 16.0	174.6 ± 22.9	31.8 ± 2.8	37.7 ± 5.9	8.0 ± 0.6
Compound II 0.02 mg/kg	507.3 ± 6.9	179.7 ± 18.0	31.9 ± 1.0	39.7 ± 4.8	7.1 ± 0.7
Compound II 0.2 mg/kg	532.3 ± 14.8	163.3 ± 12.9	27.9 ± 3.0	37.0 ± 5.3	6.7 ± 0.7
Compound II 2.0 mg/kg	570.7 ± 8.1	170.3 ± 6.6	28.0 ± 1.3	39.0 ± 3.0	6.6 ± 0.2
Compound II 20.0 mg/kg	519.0 ± 2.1	148.0 ± 20.0	27.0 ± 2.2	41.7 ± 6.9	8.3 ± 0.6

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TABLE I CONTINUED

COMPOUND I

Treatment	Body Weight (gm)	SVM Weight (mg)	Normalized SVM Weight	SVE Weight (mg)	Normalized SVE Weight
Control	483.7 ± 10.8	115.7 ± 16.1	24.3 ± 3.9	29.3 ± 4.7	6.1 ± 1.0
Compound I 0.0002 mg/kg	494.2 ± 11.0	129.8 ± 22.2	26.1 ± 4.3	33.8 ± 1.6	6.9 ± 0.4
Compound I 0.002 mg/kg	499.2 ± 10.3	115.7 ± 5.4	23.1 ± 1.1	36.7 ± 2.9	7.4 ± 0.6
Compound I 0.02 mg/kg	493.2 ± 14.5	117.3 ± 4.3	23.9 ± 1.1	34.5 ± 1.7	7.0 ± 0.3
Compound I 0.2 mg/kg	480.5 ± 8.7	130.6 ± 9.2	27.5 ± 1.7	30.0 ± 4.4	6.3 ± 1.0
Compound I 2.0 mg/kg	479.8 ± 9.7	119.3 ± 7.1	24.9 ± 1.4	35.7 ± 3.0	7.5 ± 0.7
Compound I 20.0 mg/kg	436.8 ± 7.9	126.5 ± 9.0	28.8 ± 1.4	30.8 ± 0.9	7.1 ± 0.3

The above data indicate to me that neither compound I nor compound II have any estrogenic effect in the male guinea pig. I specifically rely on the lack of stimulation of the fibromuscular stroma in making this judgment. The data have been statistically analyzed, and no significant differences are seen, except for the apparently depressed body weight caused by the highest dose of compound I.

Immature Rat Uterine Responses

I have studied and analyzed data from a set of uterotropic and antiuterotropic tests run under the supervision of Larry J. Black, and reported in his declaration which accompanies this document. The tests are identified in his system as numbers 953 and 954. A statistical analysis of that data was done at my initiative, and I have evaluated the data and drawn conclusions from it, which follow below.

Tissues from those uterotropic and antiuterotropic tests were analyzed under my supervision to determine their contents of water, total protein, DNA and RNA. Water contents were determined by simple loss of weight on drying.

Samples for the determination of DNA, RNA and protein were prepared by homogenizing a sample of about 10 mg. of tissue in 900 microliters of isotonic saline. The homogenate was split to obtain two 300 microliter samples for DNA and RNA determination, and two 150 microliter samples for protein determination. The DNA sample was diluted with 3.5 ml. of 12.5 percent aqueous trichloroacetic acid, was incubated at ice temperature (4°) for 30 minutes, and was then centrifuged. The supernatant was removed, and the solids in the centrifuge tube were diluted with 200 microliters of

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0.01N aqueous potassium acetate, remixed, incubated on ice for 15 minutes and centrifuged again. The centrifuged solids were then dried under vacuum at 70° for 1 hour, diluted with 100 microliters of 30 percent aqueous diaminobenzoic acid, incubated at 60° for 30 minutes, and read on a Perkin-Elmer fluorometer. Each set of tissues was accompanied and compared with standard samples prepared from pure DNA obtained from calf thymus. The DNA concentration of the fluorometer samples was determined by comparison with a standard curve for the instrument.

The tissue samples for RNA analysis were first diluted with 1 ml. of ice-cold 1.2N perchloric acid and mixed, incubated on ice for 15 minutes and centrifuged. The supernatant was discarded, and 1 ml. of ice-cold 0.2N perchloric acid was added to each RNA sample and mixed. The samples were incubated on ice for 15 minutes and centrifuged, and the supernatant was discarded. The 0.2N perchloric acid treatment was repeated, and the remaining solids were resuspended in 2 ml. of 0.3N potassium hydroxide and incubated for 1 hour at 37°. The samples were then chilled and 1.25 ml. of ice-cold 1.2N perchloric acid was added to each and mixed. The samples were incubated on ice for 10 minutes and centrifuged, and the supernatant was read on a spectrophotometer at 360 nM. Each set of RNA samples was compared with samples prepared in the same manner from pure RNA obtained from yeast cultures, and the RNA content of the samples was determined from a standard curve.

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Protein determinations were made by the well-known method of Lowry, which is a colorometric analysis carried out by digesting the homogenized sample with trichloroacetic acid, treating with a

copper salt and reading the color on a spectrophotometer at 750 nM.

The tissues which were studied in this work were from immature female rats, which had been treated with estradiol, compound I or II, or combinations of estradiol and compound I or II, as outlined in Table II below. Each treatment group was composed of six animals, and all treatments were administered for three days. The results of the analyses are summarized in the table below, with the body and uterine weights of the animals, as means of the various measurements for each treatment group.

It is my conclusion that the body weights of the animals treated in those tests with compound I alone were significantly lower than body weights of the control animals or those treated with estradiol alone. The ratios of RNA to DNA of the animals treated with compound I were significantly lower than the RNA/DNA ratios of the animals treated with estradiol. Compound II produced a significant increase in the normalized uterine weight and in the uterine water content, compared to the same measurements on animals treated with compound I. I therefore conclude that the difference in uterine growth caused by compound I and by compound II is more likely attributable to alterations in water content than to changes in tissue nucleic acid or protein levels.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Blake L. Neuhauer

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Date

TABLE II

RESPONSES OF IMMATURE RAT UTERI TO ESTROGEN AND TEST COMPOUNDS

	Body Weight (gm)	Tissue Weight (mg)	Tissue Weight / 100 gm body wt.	Total DNA (µg)	Total RNA (µg)	RNA / DNA	Total Protein (µg)	Percentage H_2O Content
Control	52.45 ± 1.81	23.52 ± 1.90	44.23 ± 7.18	164.40 ± 7.68	156.35 ± 29.13	0.93 ± 0.14	984.78 ± 121.55	82.70 ± 0.27
E_2 (0.1 µg.)	54.90 ± 2.12	78.58 ± 3.15	143.05 ± 10.49	236.85 ±30.33	565.40 ± 51.01	2.44 ± 0.16	3364.50 ± 117.20	84.83 ± 0.37
Compound I (1000 µg.)	46.37 ± 3.46	31.62 ± 1.53	68.66 ± 11.99	169.85 ±10.89	228.12 ± 9.79	1.37 ± 0.10	1353.50 ± 63.54	82.55 ± 0.24
E_2 + Compound I	41.83 ± 1.70	31.73 ± 1.45	75.81 ± 3.81	162.42 ±12.63	184.80 ± 15.38	1.14 ± 0.12	1252.67 ± 114.17	84.83 ± 0.35
Compound 2 (1000 µg.)	39.87 ± 1.62	36.87 ± 0.48	92.81 ± 2.94	137.78 ± 8.48	244.12 ± 15.37	1.83 ± 0.18	1516.33 ± 55.39	84.02 ± 0.37
E_2 + Compound II	44.37 ± 2.33	38.55 ± 1.90	86.96 ± 5.24	191.32 ±17.19	205.95 ± 8.99	1.11 ± 0.07	1391.17 ± 97.61	84.15 ± 0.58

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